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(71) Applicants *(for all designated States except US)*: KARO BIO AB [SE/SE]; Novum, S–141 57 Huddinge (SE). DEAN, John, Paul [GB/GB]; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2JT (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: SPYROU, Giannis [SE/SE]; Karolinska Institutet, Dept. of Biosciences at Novum, S–141 57 Huddinge (SE).

(74) Agent: DEAN, John, Paul; Withers & Rogers, 4 Dyer's Buildings, Holborn, London EC1N 2JT (GB).

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(57) Abstract

This invention relates to a thioredoxin and to methods for its use.

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MAMMALIAN THIOREDOXIN

This invention relates to the isolation and characterisation of nucleic acid encoding for a new mammalian thioredoxin and to a protein or polypeptide produced from such nucleic acid.

Thioredoxin (Trx) is a 12-kDa protein, known to be present in many prokaryotes and eukaryotes and appears to be truly ubiquitous in all living cells (Holmgren, A. (1984). Methods Enzymol. **107**, 295-300, Holmgren, A. (1985). Ann. Rev. Biochem. **54**, 237-271). It is characterized by an active site sequence -Trp-Cys-Gly-Pro-Cys-Lys-, which has been conserved throughout evolution. The active site of thioredoxin is localised in a protrusion of its three dimensional structure (Jeng. M. F. *et al* (1994) Structure **2**, 853-868) and the two cysteine residues provide the sulfhydryl groups involved in Trx-dependent reducing activity. Oxidized thioredoxin, Trx-S_2, is reduced to Trx-$(SH)_2$ by the flavoenzyme thioredoxin reductase (TR) and NADPH (the thioredoxin system) (Holmgren, A. (1985) *supra*).

Mammalian thioredoxins isolated from several sources (e.g. rat and calf liver, rabbit bone marrow and human placenta) have certain structural differences with respect to those from prokaryotes. In addition to the active site cysteine residues, two or three (depending on the Trx source) additional structural cysteine residues exist in the C-terminal half of the molecule. Oxidation of these residues leads to a loss of its enzymatic activity (Ren. X *et al.* (1993) Biochemistry **32**, 9701-9708).

More than one thioredoxin exists in many eukaryotes, e.g. yeast (Muller E. G. (1992) Yeast **8**, 117-120). However, only one thioredoxin (Trx 1) has thus far been cloned from mammalian cells.

Mammalian thioredoxin has been implicated in a wide variety of biochemical functions acting as hydrogen donor for ribonucleotide reductase (Thelander, L., Reichard, P. (1979) Ann. Rev. Biochem. **48**, 133-158) and methionine sulfoxide reductase (Holgren, A. (1985)

Supra), facilitating refolding of disulphide-containing proteins (Holmgren, A. (1988). In "Plasma Membrane oxidoreductases in Control of Animal and Plant Growth". "NATO ASI" series, eds, F.L. Grane *et al.*, Plenum Press, New York 295-302), (Lundstrom, J., Holgren, A. (1990). *J. Biol. Chem.* **265**, 9114-91120)) and activating the glucocorticoid or IL-2 receptors (Grippo, J.F., Holgren, A., Pratt, W.B. (1985) Biol. Chem. **260**, 93-97), Tagaya, *et al* (1989) EMBO J. **8**, 757-764)). It can also modulate the DNA binding activity of some transcription factors either directly (TFIIIC(9), BZLF1(10) and NF-kB(11)) or indirectly (AP-1) through the nuclear factor Ref-1 which in turn is reduced by thioredoxin (Abate, C., *et al* (1990). Science **249**, 1157-1161). The importance of the redox regulation of transcription factors by thioredoxin is exemplified by the v-*fos* oncogene where a point mutation of Cys154/Ser results in constitutive activation of the AP-1 complex (Okuno, H. *et al* (1993) Oncogene **8**, 695-701). Thioredoxin can be secreted by cells using a leaderless pathway (Ericson, M.L., *et al* (1992) Lumphokine & Cytokine Research **11**, 201-207; Rubartelli, A., *et al* (1992) *J. Biol. Chem.* **267**, 24161-24164; Rubartelli, A., *et al* (1995) Cancer Res. **55**, 675-680) and stimulate the proliferation of lymphoid cells, fibroblasts and a variety of human solid tumour cell lines (Wakasugi, N., *et al* (1990). *Proc. Natl. Acad.* SCI. USA **87**, 8282-8286; Oblong, J. E., *et al* (1994) *J. Biol. Chem* **269**, 11714-11720; Nakamura, H., *et al* (1992) Cancer **69**, 2091-2097; Gasdaska, J.R., *et al* (1995) Cell Growth & Differentiation **6**, 1643-1650). Furthermore, Trx is an essential component of the early pregnancy factor (Clarke F. M. *et al.* (1991) Reproduction & Fertility **93**, 525-539), it inhibits HIV expression in macrophages (Newman, G.W. *et al* (1994) *J. Experim. Medicine* **180**, 359-363), can reduce H_2O_2 (Spector A *et al* (1988) *J. Biol. Chem.* **263**, 4984-4990), scavenge free radicals (Schallreuter K. U., Wood J. M. (1986) *Biochem Biophys. Res. Commun.* **136**, 630-637) and protect cells against oxidative stress (Nakamura, H. *et al* (1994) Immunology Letters **42**, 75-80).

The inventors have identified a novel mammalian thioredoxin which is functionally and structurally distinct from the previously known mammalian thioredoxin. This novel thioredoxin is known as Trx2.

Accordingly, a first aspect of the invention provides an isolated nucleic acid molecule encoding the Trx2 gene product or a polypeptide which is functionally similar to Trx2.

Preferably, the nucleic acid molecule comprises the nucleic acid sequence shown in Figure 1A or Figure 9.

The nucleic acid molecule may be cDNA.

Preferably the nucleic acid has at least 60%, 70% or 80% homology, more preferably 90% homology, to the nucleic acid sequences of Figure 1A or Figure 9 encoding the Trx2 gene product or a polypeptide which is functionally similar to Trx2. This allows, for example, for variations in the sequence which still allows the production of Trx2, by virtue of degeneracy of the genetic code.

It is intended that the term "functionally similar to Trx2" means the polypeptide has thioredoxin activity, as indicated, for example by the catalysis of disulphide reduction or insulin with NADPH in the presence of mammalian thioredoxin reductase which is resistant against oxidation compared to Trx1. The known Trx1 exhibits oxidation of cysteine residues which results in a loss of its enzymatic activity (Ren X *et al.* (1993) Biochemistry **32**, 9701-9708). Trx2, however, lacks these cysteine residues.

A second aspect of the invention provides polypeptides, preferably mature proteins, encoded by nucleic acid according to the first aspect of the invention. Preferably, the polypeptide comprises the amino acid sequence of Figure 1A or Figure 10.

Analysis of the amino acid sequence of Trx2 indicates that it may be processed into a mature protein by enzymatic action. Accordingly, the invention also provides a mature protein, fragment, homologue or analogue which is at least functionally similar to Trx2. The invention also includes recombinant or synthetic polypeptides having the same or preferably better, functionality compared to native Trx2.

Preferably the mature protein comprises the amino acid sequence of Figure 1A from Thr 59 to Asp 166, or the sequence of Figure 10 from Leu 59 to Ile 166.

A further aspect of the invention provides plasmids or other vectors comprising nucleic acids according to the first aspect of the invention. Such plasmids or other vectors are preferably expression vectors of the sort known in the art, into which a nucleic acid according to the invention can be inserted.

Such plasmids or vectors may be inserted into a suitable microbial host, such as *E.coli*, or a suitable cell of the type known in the art for expression of the nucleic acid sequences of the invention such as a mammalian or insect cell. Accordingly, the invention provides bacterial, mammalian or insect cells comprising vectors or plasmids according to the invention.

Thioredoxin 2 (Trx2) is advantageous in that it is resistant to oxidation, and is translocated to mitochondria. Mitochondria are the sites of vital cellular functions such as lipid metabolism and aerobic respiration (oxidative phosphorylation). In respiration, incomplete reduction of dioxygen results in the formation of reactive oxygen intermediates, ROIs, (hydrogen peroxide, the superoxide anion O_{2^-} and the hydroxyl radical OH). Increased levels of ROIs, referred to as oxidative stress, can result in lipid peroxidation, inactivation of proteins and strand breakage in DNA. Thioredoxin can: act as an antioxidative molecule and scavenge hydroxyl radicals, reduce hydrogen peroxide and reactivate proteins inactivated by oxidation.

Accordingly the invention provides therapeutic applications of Trx2 including protection against oxidative stress induced cytotoxicity and tissue damage. More specifically Trx2 may be used to protect against ischaemic damage, eye disease, radiation, and drug toxicity.

In relation to the treatment of ischaemic damage of organs, active oxygen species are thought to play a part in ischaemia reperfusion injury. In many heart attacks and strokes an ischaemic period is followed by a reperfusion period during which oxygen radicals are produced and probably are the true cause for heart attack and stroke damage. Trx2 may be used as protective compound to attenuate ischaemia reperfusion injury.

In relation to the treatment of eye diseases, many diseases of the eye including cataract involve photo-oxidative stress. Trx2 may be used for protection of retinal cells from damage caused by active oxygen species generated during oxygen stress to the retina.

In relation to the treatment of radiation, exposure to radiation is a major source of oxygen radical oxidation. Trx2 may be used as a radio-protective compound to attenuate the effects of radiation.

Finally, in relation to the treatment of drug toxicity, several commonly used drugs (eg. adriamycin) exhibit a variety of undesirable side effects caused by generation of reactive oxygen species. For example, in the use of adriamycin, where cardiotoxicity is the major form of damage, Trx2 can be used to eliminate side effects.

The invention will now be described by way of example only, and with reference to the accompanying Figures 1-15 in which:

Figure 1 shows cDNA, deduced amino acid sequence and predicted secondary structure of rat Trx2. A) The deduced amino acid sequence in the single letter code is shown under the nucleotide sequence. The active site is boxed and the potential polyadenylation signal is underlined. B) Secondary structure was predicted using the DNASTAR program and the Garnier-Robson algorithm. The Kyte-Doolittle algorithm was applied for the hydropathy blot of Trx2 amino acid sequence;

Figure 2 shows *in vitro* translation of Trx2 cDNA using the Sp6 RNA polymerase, the TNT coupled reticulocyte transcription translation system and [^{35}S] methionine. The product was analyzed by SDS-PAGE;

Figure 3 shows alignment of the predicted amino acid sequence of rat Trx2 with that of human and rat Trx1. A) Analysis of the N-terminal part of the predicted rat Trx2 amino acid sequence. Secondary structure prediction of the region suggests an α-helix as indicated. The arrow indicates the probable mitochondrial signal peptide protease cleavage site as determined by the consensus motif for the cleavage by the two protease model

(Hendrick J. P., Hodges, P.E., Rosenberg, L.E. (1989) *Proc. Natl. Acad* SCI. USA **86**, 4056-4060). In bold are; arginine at -10; hydrophobic residues at position -8; residues S, G and T present in position -5. B) The alignment is based on a three-dimensional structure of *E.coli* thioredoxin (Eklund, H., Gleason, F.K., Holmgren, A. (1991) Proteins **11**, 13-28). Black boxes indicate conserved amino acid residues in human Trx, rat Trx1 and rat Trx2. The structure contains four α-helices, a distorted 3_{10} helix and five ß-sheets as indicated below the amino acid sequence;

Figure 4 shows a phylogenetic tree. The phylogenetic tree was constructed by the Clustal method with the PAM250 residue weight table;

Figure 5 shows Northern blot analysis of Trx1 and Trx2 in rat tissues. Each lane contains 2 µg poly(A)+ RNA. The blot was hybridized with the Trx1 and Trx2 probes as described under "Experimental Procedures". Tissues used for analysis are shown at the top. The estimated size of the mRNAs was 0.6 kb and 1.3 kb for Trx1 and Trx2, respectively;

Figure 6 shows RT-PCR analysis of transcripts encoding Trx1, and Trx2. One µg of total RNA was reverse transcribed and one µl was used as template for PCR with specific primers for Trx1, Trx2 and ß-actin. The products were separated by agarose gel electrophoresis, transferred to membranes and probed with specific oligonucleotide probes;

Figure 7 shows immunoblotting analysis of cell fractions. Cell fractions were analyzed by SDS/PAGE and developed with anti-Trx2 antibodies. Lane 1, Δ-Trx2 (5ng). Lane 2, total rat liver cell extract (15 µg), Lane 3, cytosolic fraction (15 µg), Lane 4, mitochondrial fraction (15 µg). Lane 5, peroxisomal fraction (15 µg). Lane 6, human Trx1 (5ng);

Figure 8 shows analysis of Trx activity. Oxidized Trx2 and human thioredoxin were assayed for their ability to reduce insulin in the presence or absence of DTT. The reaction was stopped after 20 min by the addition of 6 M guanidine-HCl, 1mM DTNB;

Figure 9 shows human Trx2 cDNA sequence;

Figure 10 shows the predicted human Trx2 amino acid sequence based on the cDNA sequence of Fig. 9;

Figure 11 shows two human liver cDNA sequences that code for proteins which interact with Trx2;

Figure 12 shows five rat brain cDNA sequences that code for proteins which interact with Trx2;

Figure 13 shows the human trx2 gene;

Figure 14 shows a gel picture of a PCR product in chromosome 22; and

Figure 15 shows the mRNA levels of the human Trx2 analysed by using an RNA master blot (Clontech).

1. Cloning of rat thioredoxin 2

The primary structure of the active site of thioredoxin (VVVDFSATWCGPCK), which is conserved throughout evolution was used to design degenerate primers which were labelled with ^{32}P and used as probes for screening a rat heart cDNA library (Clontech) for novel thioredoxin genes. Approximately $1x10^6$ plaques were screened according the instructions of the manufacturer (Amersham) and a positive bacteriophage was isolated. The insert from the bacteriophage was excised, cloned into the TA-vector (Invitrogen) and sequenced. A 392 bp portion of the above clone was amplified by PCR (30 cycles at; 94°C for 1 min, 52°C for 1 min and 72°C for 1 min) with specific primers (trx2f1: 5'-AACCTTTATCGTCCAGGATGGAC-3' and trx2r1: 5'-GCTGGGAGTTCTACTAGGTTCC-3').

The PCR product was ^{32}P-labelled by random priming and used to rescreen the same library under high-stringency conditions. Hybridization was performed at 60°C in

Expresshyb hybridization solution (Clontech), followed by five 10 min washes in 2 x SSC-0.1%SDS (1 x SSC is 0.15M NaCl plus 0.15M sodium citrate) at room temperature and finally two 40 min washes in 0.1%SSC-0.1%SDS at 60°C. More than 600,000 clones were screened and 5 clones were isolated, cloned into the TA-vector and sequenced. All clones were overlapping and the longest one, Trx2, possessed an open reading frame of 501 bp beginning with an ATG initiation codon and ending with a TGA termination codon. To obtain the full length cDNA 5' Rapid Amplification of cDNA Ends (RACE) with nested PCR using oligonucleotide-anchored heart cDNA template (Clontech) in the presence of an anchor-specific primer and an antisense primer complementary to the 3' untranslated region of Trx2 (trx2r2: 5'-GCTGGGAGTTCTACTAGGTTCC-3') was performed as described in the Clontech protocol. PCR products were cloned into the TA-vector and sequenced. A cDNA fragment was amplified that overlapped Trx2 and encoded 46 bp of a novel 5' sequence including an in frame TGA stop codon upstream of the ATG initiation codon. The overall composite sequence consists of 1276 bp, including a stretch of 20 adenosines corresponding to the poly(A) tail and an AATAAAA motif, 18 bp upstream from the poly(A) tail. The open reading frame encodes a protein of 166 amino acids with a predicted mass of 18.2 kDa and a pI of 7.9 (Figures 1A, 1B).

In order to confirm that the open reading frame sequence present in the Trx2 clone is functional and codes for translatable protein, the cDNA was transcribed from the SP6 promoter of the TA-Trx2 clone and 0.5 µg was translated using the TNT coupled reticulocyte lysate system (Promega) and Sp6 RNA polymerase with incorporation of [^{35}S]methionine for 60 min at 30°C. The translation products were analyzed by 15% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The results showed a 20-kDa translation product, indicating the presence of translatable, functional coding sequence (Figure 2).

2. Analysis of the deduced amino acid sequence

The N-terminal region of the protein has a high content of positively charged residues and secondary structure prediction indicated a potential α-helix followed by ß-sheets (Figure 1B).

These features are common to most mitochondrial targeting signal peptides (Neupert, W. (1994). Clinical Investigator **72**, 251-261) and an algorithm analysis of the partial amino acid composition, indicated mitochondrial intracellular localization (Newman, G. W. *et al* (1994). *J. Experim. Medicine* **180**, 359-363). A motif for mitochondrial prepeptide proteases (Hendrick J. P., Hodges, P.E., Rosenberg, L.E. (1989) *Proc. Natl. Acad.* SCI. USA. **86**, 4056-4060) was also found with the cleavage site between Ser 58 and Thr 59 (Figure 3A). This putative cleavage results in a 12.2 kDa mature protein, which is similar in size to previously reported thioredoxins.

The C-terminal half of the protein contained the active site found in all thioredoxins with the characteristic amino acid sequence, Trp-Cys-Gly-Pro-Cys-Lys. The molecule showed a 35% homology with other mammalian thioredoxins and many of the structural amino acids that are conserved in mammalian thioredoxins, i.e Phe-12, Pro-40, Asp-59, Lys-82, were also conserved in Trx2 (Figure 3B). However, amino acids participating in protein-protein interactions such as Ala-93 and Glu-57 are changed to Ile and Lys, respectively. One major difference between Trx2 and mammalian thioredoxins is the absence of structural cysteines, residues which are present in all mammalian thioredoxins.

Trx2 has higher homology with the *E.coli* thioredoxin than with the known mammalian proteins and a phylogenetic analysis places Trx2 in a different branch of the tree, distant from the mammalian proteins and closer to the prokaryotic and lower eukaryotic ones.

Sequence relatedness is summarized in Figure 4.

3. Northern blot and RT-PCR analyses of Trx1 and Trx2 mRNA expression

A rat multiple tissue northern blot with 2 μg/lane of highly pure poly (A) + RNA from different rat tissues (Clontech) was hybridized with two probes, one of 360 bp specific for rat thioredoxin (Trx1) and one of 392 bp specific for Trx2 as described in Spyrou *et al* (1996) *J. Biol Chem* **272**, 2936-2941. Rat Trx1 and Trx2 open reading frame probes were labelled with [^{32}P]dCTP by a random priming procedure and hybridized in ExpressHyb solution (Clontech). The Trx1 probe hybridized to an mRNA of 0.6 kb with highest levels in lung, liver and kidney (Figure 5). The Trx2 mRNA was detected as a 1.3 kb band, in

agreement with the size of cDNA (1,276 bp) and it was highly expressed in heart, liver, skeletal muscle and kidney. RT-PCR was used to compare the relative expression of Trx1 and Trx2 in tissues where the expression of these proteins may be very low. (Figure 6). For RT-PCR analysis, male and female rats (6-8 weeks old) were killed by cervical dislocation, tissues were collected and samples were immediately processed for total RNA isolation according to the acid guanidium thiocyanate-phenol-chloroform single step extraction protocol (Chomczynski, P., Sacchi, N. (1987) *J. Biol. Chem.* **162**, 156-159). The integrity and quality of the purified RNA was controlled by formaldehyde denaturing agarose gel electrophoresis and by measuring the A_{260}/A_{280} ratio.

For first strand synthesis, total RNA (1µg) was dissolved in 10 µl water, heated to 70°C for 5 min and then chilled on ice. The volume was increased to 20 µl, giving a final concentration of 1 mM each dATP dGTP, dCTP, dTTP, 10 mM DTT, 5 pmol random hexamers/l (Promega), 1 U RNAsin/µl, 200 U Superscript RT (GIBCO-BRL) and the incubation buffer recommended by the supplier. For PCR amplification 1 µl of cDNA (total 20 µl) was subjected to PCR and amplified for 24 cycles by incubation at 94°C for 10 sec, 54°C for 30 sec and 72°C for 60 sec in a PCR9600 thermocycler (Perkin-Elmer, Norwalk, CT). The oligonucleotides trx2f1: 5'-AACCTTTATCGTCCAGGATGGAC-3' and trx2r1: 5'-GCTGGGAGTTCTACTAGGTTCC-3' were used for the amplification of a 392 bp fragment of the Trx2 mRNA. The oligonucleotides trx1f1: 5' - CCAAAATGGTGAAGCTGATCGAGAG-3' and trx1r1: 5'-TGATTAGGCAAACTCCGTAATAGTG-3' were used for the amplification of a 360 bp fragment of the Trx1 mRNA. The oligonucleotides Act 5', CTGGCACCACACCTTCTA and Act 3', GGGCACAGTGTGGGTGAC were used for the amplification of a 238 bp fragment from ß-actin mRNA. After agarose gel electrophoresis and blotting to nitrocellulose filters the PCR products were hybridized to ^{32}P-labelled internal oligonucleotides: trx2r2: 5'-CACCACCTTCCCGTGCTGTTT-GGCTACCATCTTCTCTAACCGAGGTCC-3' for Trx2, trx1r2: 5'-CTGGAATGTT-GGCGTGCATTTGACTTCACACTCTGAAGCAACATCCTG-3' for Trx1 and actin primer 5'- GATGACCCAGATCATGTTTGA-3'.

Hybridization was performed at 50°C in Expresshyb hybridization solution followed by five 10 min washes in 2xSSC-0.1%SDS at room temperature and finally two 40 min washes in 0.1%SSC-0.1%SDS at 50°C. While Trx1 is found to follow the β-actin expression with higher expression in colon and liver, Trx2 gave a completely different pattern. It was highly expressed in cerebellum, heart, skeletal muscle, kidney, adrenal gland and testis. No cross hybridization between Trx1 and Trx2 was observed.

4. Expression of recombinant thioredoxin2 and subcellular localization

The C-terminal part of the cDNA encoding a part of rat Trx2 (aa 60-166, Trx2) was amplified by PCR from the TA-Trx2 plasmid by using two mutagenic primers that introduce a *Nde*I (trx2p1: 5'-ACCACCAGAGTCCATATGACAACCTTTAACGTC-3') and a *Bam*H I (trx2p2: 5'-CTGGCCGGATCCCTGCTTATCAGCCAATTAGC-3') site at the N-terminus and C-terminus of the polypeptide respectively. The amplified DNA was cloned into the *NdeI-Bam*HI sites of the pET-15b expression vector (Novagen) under the control of a T7 promoter, and the resulting plasmid, pET-trx2 was transformed into the *E. coli* strain BL21 (DE3). A single positive colony was innoculated in 1 l of L-broth with 50 μg/ml ampicillin and grown at 37°C until OD_{600}=0.5. Then, fusion protein expression was induced by addition of 0.5 mM IPTG and growth continued for another 3.5 h. The cells were harvested by centrifugation at 10,000 x g for 10 min, the pellet was resuspended in 50 ml of 20 mM Tris-HCl pH 8.0, 100 mM NaCl and 1 mM PMSF. Lysozyme was added to a final concentration of 0.5 mg/ml with stirring for 30 min on ice. Subsequently, $MgCl_2$ (10 mM), $MnCl_2$ (1mM), DNAse I (10 μg/ml), and RNAse (10 μg/ml) were added and the incubation was continued for another 45 min on ice. The cells were disrupted by sonication for 8 min, the supernatant was cleared by centrifugation at 15,000 x g for 30 min and loaded onto a Talon resin column (Clontech) and the protein was eluted with 20 mM imidazole. The size and purity of the eluted protein was determined by SDS-PAGE on a 15% gel. A single band of 15 kD was detected after the Talon chromatography (data not shown).

The inventors next analyzed the subcellular localization of Trx2 using affinity purified polyclonal antibodies obtained from immunized rabbits (Zeneca Research Biochemicals, England). After 6 immunizations, serum from the rabbits was purified by ammonium

sulfate precipitation. Affinity-purified antibodies were prepared using a cyanogen bromide-activated Sepharose 4B column, onto which 0.5 mg of Trx2 had been coupled using the procedure recommended by the manufacturer (Pharmacia). Specificity of the antibodies was tested by western blotting using recombinant Trx2 and total cell extracts. Mitochondrial, peroxisomal and cytosolic fractions were prepared from rat liver as described (Svensson, L.T., Alexson, S.E.H., Hiltunen, J.K. (1995) *J, Biol. Chem.* **270**, 12177-12183). For immunoblotting analysis samples were subjected to 15% SDS-PAGE and the separated proteins were electrophoretically transferred to nitrocellulose membranes (Hybond-C Super, Amersham). The membranes were blocked with PBS containing 5% dry fat free milk powder and 0.05% Tween 80 and further incubated with affinity purified anti-Trx2 antibodies. Immunodetection was performed with horseradish peroxidase-conjugated goat anti rabbit IgG (Amersham) diluted 1:5000 following the ECL protocol (Amersham) in a hydrogen peroxide catalyzed oxidation of luminol. As shown in Figure 7, Trx2 is only present in mitochondrial fractions as neither cytosolic nor peroxisome enriched fractions displayed any signal. Rat Trx1 did not cross-react with the affinity purified antibodies against Trx2. By densitometric analyses Trx2 content in total cell-free extracts from rat liver was estimated to be around 0.1 µg/mg protein (data not shown). The transient preprotein with the mitochondrial translocation peptide was not detected indicating that the translocation process is very fast. The recombinant Trx2 in lane 1 has a higher molecular weight because the His-tag was not removed by thrombin.

5. Thioredoxin catalyzed insulin reduction

In order to confirm the specificity of the recombinant Trx2, the inventors examined the reduction of insulin, a classical assay in which thioredoxin catalyzes disulfide reduction of insulin with NADPH in the presence of mammalian thioredoxin reductase (TR). They compared the activities of human thioredoxin with the recombinant Trx2. The insulin disulfide reduction assay was essentially performed as described (Holgrem, A., BjÜrnstedt, M. (1995) Methods Enzymol **252**, 199-208)with a slight modification to activate Trx1 and Trx2 by reduction. Aliquots of Trx1 and Trx2 were preincubated at 37°C for 20 min with 2 µl of: 50 mM Hepes pH 7.6, 100 µg/ml BSA and 2 mM DTT, in a total volume of 70 µl. Then, 40 µl of a reaction mixture composed of 200 µl Hepes (1M) pH 7.6, 40 µl EDTA (0.2 M), 40 µl NADPH (40 mg/ml) and 500 µl insulin (10 mg/ml) was added. The reaction

started with the addition of 10 µl of TR from calf-thymus (3.0 A_{412} unit) and incubation continued for 20 min at 37°C. The reaction was stopped by the addition of 0.5 ml of 6 M guanidine-HCl, 1mM DTNB and the absorbance at 412 nm was measured. Calf-thymus TR and human thioredoxin were kind gifts from Prof. A. Holmgren, Karolinska Institute, Sweden. As shown in Figure 8, when the samples were preincubated with DTT, Trx2 and human Trx were equally good substrates for TR. However, oxidized human Trx showed a decreased capacity to reduce insulin with a pronounced lag phase. The activity of Trx2 was not affected upon oxidation. Although Trx2 is homologous to the prokaryotic thioredoxins it could not function as a substrate for *E.coli* TR (data not shown).

6. Isolation of human cDNA

Using polymerase chain reaction, human cDNA encoding Trx2 has been isolated. This is shown in Figure 9. This is 87.4 % homologous to rat Trx2 cDNA.

The predicted amino acid sequence of human Trx2 is shown in Figure 10. This also contains a putative mitochondrial prepeptide protease cleavage site between Ser58 and Leu59.

7. Thioredoxin 2 interacting proteins

In order to determine which proteins Trx 2 interacts with, a human liver cDNA library was used to isolate Trx2 interacting proteins with the yeast two hybrid system. Trx2 was cloned into the pGBT9y vector (Clontech), expressing the DNA binding domain of GAL4 transcriptional activator fused with Trx2. The liver cDNA library was cloned into the pGAD10 vector (Clontech) that expresses the cloned cDNA fused with the DNA activation domain of GAL4. These two vectors were co-transformed to the HF7c yeast strain that is auxotrophic to tryptophan and leucine and has histidine as a reporter gene under the control of the upstream activating sequence (UAS) and TATA portion of the GAL1 promoter. The pGBT9 (Clontech) and pGAD10 vectors contain the selection markers for tryptophan and leucine respectively. Upon interaction of Trx2 with the cloned protein in the pGAD10 vector the transcriptional activation of GAL4 is restored leading to expression of histidine thus supporting growth on plates lacking histidine. Approximately

$16x10^6$ clones were examined and 200 colonies could grow on plates lacking Leu/Trp/His. For further analysis 110 positive (growing) clones were selected.

The proteins isolated were fibrinogen β-chain precursor, plasminogen, vitronectin, PDI, γ-actin, serine hydroxymetyltransferase, NF-kB p65 and two cDNA sequences that code for proteins with no apparent homology to known proteins in a protein sequence data base (Figure 11).

Using a rat brain cDNA library screened in the same way as above, the following clones were isolated: cytochrome c oxidase, rat ribosomal protein S17, c-myc intron binding protein (zinc finger protein), and five cDNA sequences that code for proteins with no apparent homology in the database. The cDNA sequences can be seen in Figure 12.

8. **Genomic organisation of Trx2**

To locate the number and position of the introns within the trx2 gene (Figure 13) of the human genome, 2 sets of primers were generated for use in the PCR amplification of the portions of trx2 gene, the first set comprising a forward primer corresponding to aa1 to aa7 and a reverse primer from aa110 to aa117 and the second set comprising a forward primer from aa88 to aa96 and a reverse primer from downstream of the stop codon. The first set when used for amplification from human genomic DNA gave a product of approximately 4.2kb and the second primer set gave a product of approximately 9bk thereby giving a combined intron total of approx. 13.2 kb. Both these products were cloned into the pGEMT easy vector (Promega) and sequenced. In the first PCT amplification product, only one intron of 4kb was present which was located between aa87 and aa88 of trx2. The 9kb PCR amplification had only one intron as well which was located between aa129 and aa130 of trx2. Both splice junctions conformed to the GT/AG rule. The trx2 gene was therefore shown to possess 2 introns.

9. Isolation of human PAC clones and Chromosomal localisation

The human PAC library (No. 704 purchased from the Resource Center/Primary Database of the German Human Genome Project, Berlin), constructed from a human male fibroblast cell line and ligated into pCYPAC-2 (Lehrach *et al.*, (1990); Loannou *et al.*, (1994)), was

screened with the genomic DNA probe H6-2. Trx2 from the human Trx2 gene. The 4.2 kb fragment was labelled with ^{32}P by random priming and hybridized to the filter bound PAC clones under stringent conditions (45% formamide, 42°C) over night. After hybridization the filters were washed 20 min in 2XSSC, 0.1% SDS and 20 min in 0.1XSSC, 0.1% SDS at room temperature, followed by three 30 min washes in 0.1XSSC, 0.1% SDS at 65°C. Autoradiography (FUJI Medical X-ray) was analyzed after 4 days. One clone of 130kb was found containing the trx2 gene from which DNA was isolated by Qiagen preparation. To further confirm that the clone is a true positive, a PCR was performed using the primer sets used above which gave the same amplification products.

10. Somatic cell hybrid panel Fluorescence in situ Hybridization (FISH)

In order to identify which human chromosome carried the trx2 gene, high molecular weight DNA was isolated from somatic hybrid cell lines (hamster/human and mouse/human) retaining individual human chromosomes. PCR amplification was carried out on each chromosome using primers corresponding to the start and stop codon. The expected product size was approx. 130kb. Chromosome 22 was found to be the one containing the trx2 gene since this was the chromosome that generated the expected product by PCR amplification (Figure 14).

The PAC clone of trx2 was used as a probe after labelling with biotin-16-dUTP by nick-translation. To obtain more detailed analyses of the localization of the trx2 gene, slides with human metaphase chromosomes were prepared using standard procedures. The slides were postfixed, Rnase treated and denatured as previously described (Pinkel *et al* (1986) *Proc. Natl. Acad. Sci* USA **83**, 2934-2938). The PAC clone for Trx2 was used as a probe after labelling with biotin-16-dUTP by nick-translation. The probe (50ng, 100ng) was pre-annealed with Cot-1 DNA (2.5-3.5 (g) for 30-60 min at 37°C after denaturing in 68°C for 10 min. Hybridization was performed in 50% formamide at 37°C overnight in a moist chamber. The slides were then washed three times for 5 min in 50% formamide, 2XSSC at 42•C and three times in 0.1XSSC at 60•C. After washing, the hybridized probe was coupled to fluorescein-isothiocyanate-avidin D and the fluorescent signal was amplified by three successive treatments with biotinylated anti-avidin antibodies alternated with fluorescein-isothiocyanate-avidin D (Vector Lab). After dehydration, the slides were mounted in glycerol containing

2.3% DABCO (1,4-diazabicyclo- (2,2,2) octane) as antifade, and DAPI (4,6-diamino-2-phenyl-indole) at 0.5(g/ml as counterstain. The signal was visualized using a Zeiss Axioskop fluorescence microscope equipped with a cooled CCD-camera (Photometrics Sensys) controlled by a Power Macintosh Quadra 950 computer. Gray scale images were captured, pseudocolored and merged using the SmartCapture software (Vysis). The hybridisation signal was detected as symmetrical spots on both chromatids of the homologues chromosomes 22 at q131.1.

11. Northern analysis of Trx2 expression

To detect the presence of trx2 mRNA molecules within human cell types, a trx2 cDNA probe labelled with ^{32}P dUTP was hybridized to a human mRNA master blot (Clontech). After exposing the blot to autoradiography film, densitometric measurements were performed on the dots appearing on the film. The values measured were then corrected with G3PDH (glyceraldehyde-3-phosphate dehydrogenase). The highest value was set as 100% and the rest were then compared to the set value. The relative values for the various tissue mRNAs blotted can be seen in Fig 15.

12. Submitochondrial localization and immunohistochemical analysis

To better understand the function of Trx2 in mitochondria and evaluate the interaction of Trx2 with some of the proteins isolated in the two hybrid screening it is necessary to elucidate its submitochondrial localization of Trx2. Using perfused rat kidney and immunohistochemistry in combination with electron microscopy, the inventors showed that trx2 is located in the inner mitochondrial membrane.

Immunocytochemistry

For light microscopic immunocytochemistry the rats were anesthesized with pentobarbital and perfused transcardially first with 100ml of saline followed by a mixture of 4% paraformaldehyde and 0,1% picric acid in 0.1 M phosphate buffered saline (PBS; pH. 7.3) for 4-5 minutes. After perfusion the tissues were excised and further fixed by immersion in the same solution for 60 min. The samples were cryoprotected with 15% sucrose in PBS

before sectioning in the cryostat. Immunocytochemistry was performed using ABC-method. The sections were first incubated with rabbit antiserum against Trx2 (dil. 1:250 in PBS containing 1% BSA and 0.3% Triton X-100) for 12-24h. After several washes the sections were incubated with biotinylated goat-anti-rabbit (Vector Labs, dil. 1:300) and ABC-complex for 30 min each. Diaminobenzidine was used as a chromogen to visualize the sites expressing Trx2-immunoreactivity. The sections were dehydrated, mounted and examined with Nikon Microphot-FXA microscope.

Immunoelectron microscopy

For immunoelectron microscopy the anesthesized animals were perfused with a mixture of 4% paraformaldehyde, 0.1% picric acid and 0.2% glutaraldehyde in PBS for 5 min. The tissues were postfixed by immersion for 5-6 h. The tissues were cryoprotected with 50% sucrose and 10% glycerol in PBS for several days. The tissues were rapidly frozen in liquid nitrogen and 50um frozen sections were cut with the cryostat. The sections were processed free floating. The Trx2 antibody was diluted (1:50) with PBS containing 1% BSA and 0.2% Saponin and the sections were incubated for 4-6 days with the antibody. After several washes the sections were incubated with secondary antibody and ABC-complex for 12 h each. Diaminobenzidine was used as chromogen. Subsequently the section were fixed with 2% glutaraldehyde, 1% osmium tetraoxide and 1% uranyl acetate for 30 min each. The tissues were dehydrated with ethanol and embedded in Epon. The samples were processed for electron microscopy and the thin sections were examined in Jeol 1200 electron microscope.

Nitric acid (NO) is a short lived free radical gas with a variety of physiological roles which include S-nitrosylation of proteins *in vivo*. Since Thioredoxin 1 is well known to scavenge free radicals (Schallreuter, K.V., Wood J. M. (1986) *Biochem. Biophys. Res. Commun* **136**, 630-637) and can reverse the action of NO in AP1 *in vitro*. (Nikitorite *et al* (1998) *Biochem. Biophys Res. Commun.* **242**, 109-112), it is therefore possible that trx2 located in mitochandria may have a similar function to trx1, thus reversing the effect of NO on mitochondrial proteins.

13. Immunocytochemistry and *in situ* hybridization in different tissues of adult male and female rat

In situ hybridization

In situ hybridization was carried out as described in detail previously(Kononen and Pelto-Huikko, 1997, TIPS online). Two oligonucleotides (nucleotides trx2r2:CACCACCTTCCCGTGCTGTTTGGCTACCATCTTCTCTAACCGAGGTCC and trx2r3: GACAACTCTGTCTTGAAAGTCAGGTCCATCCTGGACGTTAAAGGTTGT) specific for rat Trx2 were labeled to a specific activity of 1 x 109 cpm/mg at the 3'-end with 33P-dATP (NEN, Boston, MA) using terminal deoxynucleotidetransferase (Amersham). Both probes produced similar results when used separately and were usually used together in the hybridizations to increase the intensity of the signal. Several control probes with similar CG-content and specific activity were used to ascertain the specificity of the hybridizations. Addition of 100 molar excess of the unlabeled probes abolished all hybridization signals. Rats were decapitated and the tissues excised and frozen on dry ice. Cryostat sections (14um) were sectioned with Micron HM500 cryostat and thawed onto Probe-On glass slides (Fisher Scientific, Philadelphia, PA). The sections were stored at -20°C until use. The sections were incubated in humidified boxes at 42°C for 18h with 5ng/ml of the labeled probe in the hybridization cocktail, washed, dried and covered with Amersham B-max autoradiograph film for 30-60 days. Alternatively the sections were dipped in Kodak NTB2 nuclear track emulsion (Rochester, NY) and exposed for 90 days at 4°C. The sections were examined in a Nikon Microphot-FXA microscope equipped for dark-field and epipolarization microscopy. Finally, the sections were stained with cresyl violet and analyzed under brightfield conditions.

The results of the immunocytochemical and *in situ* hybridization studies are shown below:

Male Genital organs:

Testis:

In situ: high levels of trx2 mRNA in seminiferous tubules, variation during spermatogenic cycle.

Immuno: Strong labelling in Sertoli cells, primary spermatocytes and also some labelling in Leydig cells.

Epididymis:

In situ: Signal in the epithelium.

Immuno: The epithelial cells are clearly labelled.

Prostate:

In situ: Clear signal in epithelium.

Immuno: Most of the epithelial cells labelled.

Female genital organs:

In situ: Strong signal in ovary. Corpus lutea are strongest, also labelling in follicles and interstitium. Signal in epithelium and muscula layer in uterus.

Immuno: Strong labelling in collapsing follicles. Some labelling in corpus luteum. Labelling in Oocytes in primordial follicles. Clear signal in epithelium in uterus and also staining in smooth muscle cells in myometrium.

Skin

Immuno: Labelling in keratinocytes in the basal epidermis. Labelling in some cells in hair follicles. Labelling in epithelial cells in sweat glands.

Endocrine organs:

Pituitary: Moderate labelling with immuno in large number of cells, low signal with *in situ.*

Adrenal gland: Strong signal with *in situ* in the adrenal cortex, low signal in medulla. Strong labelling in adrenal cortex, especially zona glomerulosa and fasciculata. No staining in chromaffin cells in medulla.

Alimentary tract:

Salivary gland: Some labelled cells in secretory alveoli.

Stomach: Strong labelling in the mucosa, in parietal cells (secrete acid) of gastric glands and in the epithelial cells of the mucosa.

Duodenum: Strong labelling in epithelial cells in the mucosa.

Liver: Uniform clear signal with *in situ*. Clear labelling in most of the hepatocytes, some are very strongly stained.

Eye:

Clear staining in cornea; in epithelium, stromal fibroblasts and endothelium.

Lens: Very strong staining in subcapsular epithelium of the lens.

Retina: Staining in ganglion neurons and in bipolar neurons (rods and cones negative). Labelling in pigment epithelium.

Respiratory tract:

Staining in several cells at alveolar level. Parasympathetic neurons in local ganglia are immunoreactive.

Kidney:

Strong signal with *in situ* in the medulla of kidney (loops of Henle at this area), lower labelling in the papilla (collecting tubules at this area). Labelling also in cortex.
With immuno clear signal in tubules (Loops of Henle) in the medulla, in collecting tubules. Labelling also in glomeruli.

Immune system:

Clear signal with in lymph nodes, spleen, thymus and bone marrow. Strong staining in large number of lymphocytes (proliferating cells in germinal centres) in lymph nodes with immuno. Many cells stained both in spleen and in thymus. In bone marrow several celltypes, megacaryocytes are easily identified.

Brown fat:

Strong labelling with immuno especially in young animals.

Bone:

Labelling in chondrocytes in cartilage. Labelling in cells lining bone. Strong labelling in some cells around developing bone. With *in situ* very strong signal in some cells in developing bone.

Muscle:

Labelling in large number of cells in muscles.

Peripheral nervous system:

Large number of labelled neurons in sympathetic ganglia, satellite cells (glia) negative. Part of the cells in carotid body (chemoreseptor) are immunoreactive. Motoneurons in spinal cord are labelled. Large number of the sensory neurons labelled, strongest labelling in small neurons (related to pain) and middle-sized neurons.

Central nervous system:

Trx2 is mainly localised in special sets of neurons (mitral cells in olfactory bulb, magnocellular neurons in supraoptic nucleus, substantia nigra, Purkinje cells).

Circulatory system:

Heart: *In situ*: Strong signal both in atrium and in ventrical (in cardiac muscle).
Immuno: Strong staining in most of the muscle cells.

Blood vessels: Clear staining of the smooth muscle cells and in small blood vessels.

From previous studies is known that smooth muscle cells do no contain any Trx1. Considering the antioxidant function of thioredoxins, the possibility of a correlation of trx2 expression and artheriosclerosis is very interesting.

These results demonstrate the prolific nature of the trx2 mRNA and translation product within the tissues of the adult male and female rat.

In particular, the very strong staining in the subcapsular epithelium of the lens of the eye suggests an important role of Trx2 in the protection of the lens caused by active oxygen, since thioredoxin has been shown to protect cells against oxidative stress (Nakamura, H. *et al* (1994). Immunology Letters **42**, 75-80).

Similarly, the high level of expression of Trx2 in neurons suggests an important role for Trx2 in protecting these cells from oxidative damage. For example, the high expression of Trx2 in neurons together with the ability of thioredoxins to scavenge free radicals and regenerate damage proteins suggests Trx2 may have a role in the limitation and/or reversal of neuron damage since a common factor in a variety of neurodegenerative diseases is the production of free radicals.

Trx2 may also play a significant role in the control of pain, since there are a large number of pain-related sensory neurons in the peripheral nervous system which are strongly labelled with the Trx2 antibody.

14. Comparison of Trx2 with known Trx1 proteins

A Southern hybridization analysis of the human genome suggested several thioredoxin genes, including at least one inactive pseudogene (Tonissen, K. F., Wells, J. R. (1991) Gene **102**, 221-228).

The encoded protein sequence of Trx2 shows an interesting two-domain structure consisting of a N-terminal part of a 60 amino acid region rich in basic amino acids with a theoretical pI of 12.1 and a C-terminal part homologous to thioredoxin with a pI of 4.8. The N-terminal of Trx2 has characteristic properties of a mitochondrial translocation peptide and a proposed protease cleavage site which may give a mature protein of 12.2 kD. In fact, a slightly larger mitochondrial form of Trx, compared to the cytosolic Trx, has been reported to be present in pig heart based on electrophoretic mobility (Bodenstein, J., Follman, H. (1990) Z. Naturforsch **46c**, 270-279). *In vitro* coupled transcription/ translation confirmed the presence of the putative open reading frame in the Trx2 clone. The size of the translation products in the SDS-polyacrylamide gel electrophoresis analysis was somewhat larger than the calculated sizes (20 kDa versus 18.2 kDa), but this may be

due to the primary characteristic of the protein (eg. charge). Of course, the native protein may still have a different size due to post-translational modification.

Although Trx2 is phylogenetically closer to prokaryotic than mammalian thioredoxin some amino acids conserved in all prokaryotes like Trp-28 are not conserved in Trx2. Also the differences in amino acids involved in protein interaction such as Ala-93 and Glu-57 will probably confer a different specificity for Trx2 compared to Trx1. All previously described mammalian thioredoxins have 2-3 additional cysteine residues to the two located in the active site. These structural or non catalytic cysteine residues can undergo oxidation, a process which leads to inactivation. From the structure of reduced human thioredoxin Cys-72 is located in a loop in proximity to the active site. Xilin et al. (Ren, X. *et al* (1993) Biochemistry **32**, 9701-9708) showed that Cys-72 is responsible for dimer formation and subsequent loss of activity. The absence of corresponding structural cysteines in Trx2 confers a resistance to oxidation. This property might have important physiological implications for the role of Trx2.

Mammalian Trx can be found in many different cellular compartments including nucleus, endoplasmic reticulum, mitochondria and plasma membrane (Martin, H., Dean, M. (1991) *Biochem. Biophys. Res. Commun* **175**, 123-128), (Holgrem, A., Luthman, M. (1978) Biochemistry **17**, 4071-4077)). Also Trx is differentially regulated and has separate functions including promotion of cell growth to transcription factor activation and radical scavenging activities. Trx2 is highly expressed in tissues such as heart and skeletal muscle where Trx1 protein is not detectable (Fujii, S. *et al* (1991). Virchows Archiv A, Pathological Anatomy & Histopathology **419**, 317-326). Reactive oxygen intermediates (ROI), which comprise hydrogen peroxide, hydroxyl radicals and superoxide anions, are essential compounds of oxidative metabolism (Halliwell, B., Gutteridge, J.M.C. (1990) Methods Enzymol **186**, 1-85). An important source of ROI are mitochondria (Turrens, J. F., Boveris, R. (1980). *Biochem. J.* **191**, 421-427). Generally, ROI are regarded as toxic and harmful metabolites and when their formation occurs in an uncontrolled fashion, they may be implicated in several diseases by inducing lipid peroxidation and disruption of structural proteins, enzymes and nucleic acids. Recently, ROI in addition to being cytotoxic, have been reported to function as signal transducers of TNF-induced gene

expression (Schulze, O.K. *et al* (1993) *EMBO. J.* **12**, 3095-3104). Thioredoxin can reduce hydrogen peroxide and scavenge free radicals (23-25). Using affinity purified antibodies against Trx2 the inventors showed that it is localised in the mitochondria and the mature protein has an apparent molecular weight of 13 kD. A mitochondrial localised Trx2, which is more resistant to oxidation than Trx1, may explain its high expression in heart and skeletal muscle, tissues with high metabolic activity, and confer an important regulatory and/or protective function.

CLAIMS

1. An isolated nucleic acid molecule comprising at least a portion of the nucleic acid sequences shown in Figure 1A or Figure 9.

2. A nucleic acid molecule according to claim 1, wherein the nucleic acid molecule is cDNA.

3. A nucleic acid molecule demonstrating greater than 87.4% homology to a nucleic acid molecule according to claim 1 or 2.

4. An isolated nucleic acid molecule according to any of claims 1 to 3 wherein the isolated nucleic acid molecule is from a mammal.

5. An isolated nucleic acid molecule according to claim 4 wherein the mammal is a rat.

6. An isolated nucleic acid molecule according to claim 4 wherein the mammal is a human.

7. An isolated human nucleic acid molecule obtained using a nucleic acid molecule according to any of claims 1 to 5.

8. A protein or polypeptide encoded by a nucleic acid molecule according to any previous claim.

9. A protein or polypeptide according to claim 8 comprising at least a portion of the amino acid sequence shown in Figure 1A or Figure 10.

10. A composition comprising a protein or polypeptide according to either of claims 8 or 9 wherein the protein or polypeptide has the ability to scavenge free radicals.

11. A composition comprising a protein or polypeptide according to any of claims 8 to 10 wherein the protein or polypeptide has the ability to regenerate damage proteins.

12. A composition comprising a protein or polypeptide according to any of claims 8 to 11 wherein the protein or polypeptide is involved in the control of pain within an organism.

13. A composition comprising a protein or polypeptide according to any of claims 8 to 12 wherein the protein or polypeptide provides protection against oxidative stress induced cytotoxicity and tissue damage.

14. A composition comprising a protein or polypeptide according to any of claims 8 to 13 wherein the protein or polypeptide provides protection against ischaemic damage to organs.

15. A composition comprising a protein or polypeptide according to claim 14 wherein the protein or polypeptide can attenuate ischaemic reperfusion injury.

16. A composition comprising a protein or polypeptide according to any of claims 8 to 15 wherein the protein or polypeptide comprises a protective compound against heart attacks and strokes.

17. A composition comprising a protein or polypeptide according to any of claims 8 to 16 wherein the protein or polypeptide provides protection to retinal cells from damage caused by active oxygen species generated during oxygen stress to the retina.

18. A composition comprising a protein or polypeptide according to any of claims 8 to 17 wherein the protein or polypeptide comprises a radio-protective compound which attenuates the effects of radiation.

19. A composition comprising a protein or polypeptide according to any of claims 8 to 18 wherein the protein or polypeptide protects neuronal cells from oxidative damage.

20. A protein or polypeptide according to any of claims 8 to 19 wherein the protein or polypeptide can limit or eliminate the side effects of medicinal drugs caused by generation of reactive oxygen species.

21. A mature protein, fragment, homologue or analogue of a protein according to claims 8 to 20 which mature protein, fragment, homologue or analogue is at least functionally similar to Trx2.

22. A mature protein according to claim 21 comprising the amino acid sequence of Figure 1A from Thr 59 to amino acid 166.

23. A mature protein according to claim 21, comprising the amino acid sequence of Figure 10 from Leu 59 to amino acid 166.

24. A plasmid or other vector comprising a nucleic acid molecule according to any one of claims 1 to 7.

25. A host cell containing a plasmid or other vector according to claim 24.

26. A host cell according to claim 25 which is a mammalian, bacterial or insect cell.

27. Antibodies raised against a protein or polypeptide according to any of claims 8 to 23.

28. A diagnostic probe wherein the probe comprises any portion of the protein or polypeptide according to any of claims 8 to 23.

29. Diagnostic tests, assays or monitoring methods using a protein or polypeptide or any fragments of a protein or polypeptide according to any of claims 8 to 23.

30. Diagnostic tests, assays or monitoring methods using a probe comprising at least a portion of a nucleic acid molecule according to any of claims 1 to 7.

31. Diagnostic tests, assays or monitoring methods according to either of claims 29 or 30 wherein the tests, assays or monitoring methods comprise microbiological, animal cell, or biodiagnostic tests, assays, and monitoring methods.

32. The production of a protein or polypeptide according to any of claims 8 to 23 by chemical or biological means.

33. An organism engineered to contain or overexpress the protein or polypeptide according to any of claims 8 to 23.

34. An organism engineered to produce Trx2 encoded by a nucleic acid molecule according to any of claims 1 to 7.

35. An organism according to either of claims 33 or 34 wherein the organism is selected from bacteria or yeast.

36. An organism according to either of claims 33 or 34 wherein the organism is selected from rat and human species.

37. A method of treating a subject with ischaemic damage or retinal damage, the method comprising supplying to the subject a pharmaceutical composition comprising a protein or polypeptide according to any one of claims 8 to 23.

38. A method of treating a subject with oxidative stress induced cytotoxicity and tissue damage, the method comprising supplying to the subject a pharmaceutical composition comprising a protein or polypeptide according to any one of claims 8 to 23.

39. A method of controlling pain within a subject, the method comprising supplying to the subject a pharmaceutical composition comprising a protein or polypeptide according to any one of claims 8 to 23.

40. A method of protecting against damage caused by heart attacks and strokes in a subject, the method comprising supplying to the subject a pharmaceutical composition comprising a protein or polypeptide according to any one of claims 8 to 23.

Fig. 1A

```
TGA CGG GCA GGC CGG CTG GGC TCT GCA TCC CTC TGC TCA CAC TGC CGG
***
GAG ATG GCT CAG CGG CTT CTC CTG AGG AGG TTC CTG ACC TCA GTC ATC
     M   A   Q   R   L   L   L   R   R   F   L   T   S   V   I
TCC AGG AAG CCT CCT CAG GGT GTG TGG GCT TCC CTC ACC TCT ACG AGC
 S   R   K   P   P   Q   G   V   W   A   S   L   T   S   T   S
CTG CAG ACC CCT CCG TAC AAT GCT GGT GGT CTA ACT GGA ACA CCC AGC
 L   Q   T   P   P   Y   N   A   G   G   L   T   G   T   P   S
CCT GCC CGG ACA TTT CAC ACC ACC AGA GTC TGT TCA ACA ACC TTT AAC
 P   A   R   T   F   H   T   T   R   V   C   S   T   T   F   N
GTC CAG GAT GGA CCT GAC TTT CAA GAC AGA GTT GTC AAC AGT GAG ACA
 V   Q   D   G   P   D   F   Q   D   R   V   V   N   S   E   T
CCA GTT GTC GTG GAC TTT CAT GCA CAG TGG TGT GGC CCC TGC AAG ATC
 P   V   V   V   D   F   H   A   Q   W   C   G   P   C   K   I
CTA GGA CCT CGG TTA GAG AAG ATG GTA GCC AAA CAG CAC GGG AAG GTG
 L   G   P   R   L   E   K   M   V   A   K   Q   H   G   K   V
GTG ATG GCC AAA GTG GAC ATT GAC GAT CAC ACA GAC CTT GCC ATT GAG
 V   M   A   K   V   D   I   D   D   H   T   D   L   A   I   E
TAC GAG GTG TCT GCT GTG CCT ACC GTG CTG GCC ATC AAG AAC GGG GAC
 Y   E   V   S   A   V   P   T   V   L   A   I   K   N   G   D
GTG GTG GAC AAG TTT GTG GGG ATC AAG GAC GAA GAC CAG CTG GAA GCC
 V   V   D   K   F   V   G   I   K   D   E   D   Q   L   E   A
TTC CTG AAG AAG CTA ATT GGC TGA CAAGCAGGGAAGAGGCCAGACTGCCCTTGCC
 F   L   K   K   L   I   G  ***
TGCTGGAAACCCATTTGGGGAGGAACCTAGTAGAACTCCCAGCCCTCAGCTGTCATCCTTCGT
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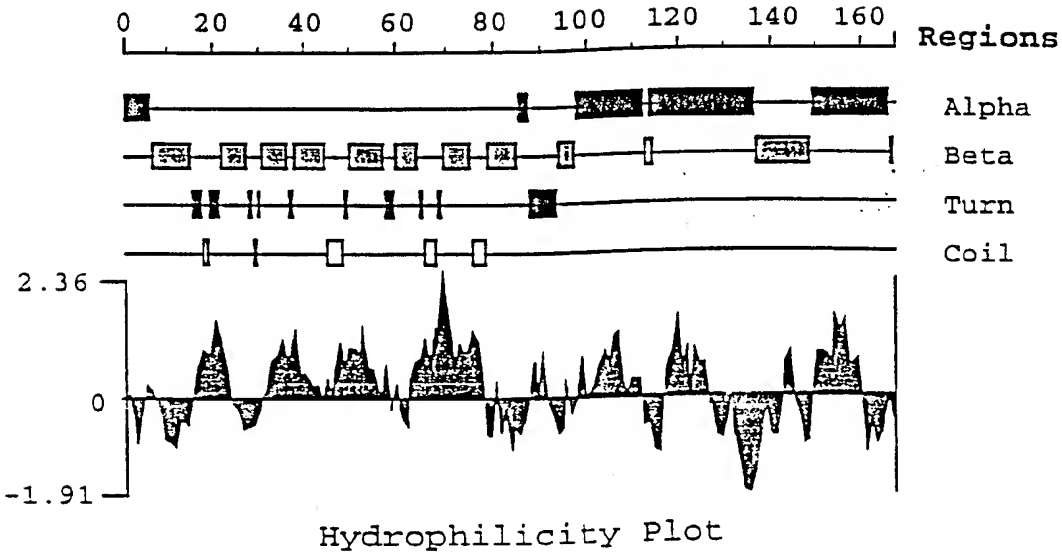
Fig. 1B


0
20
40
60
80
100
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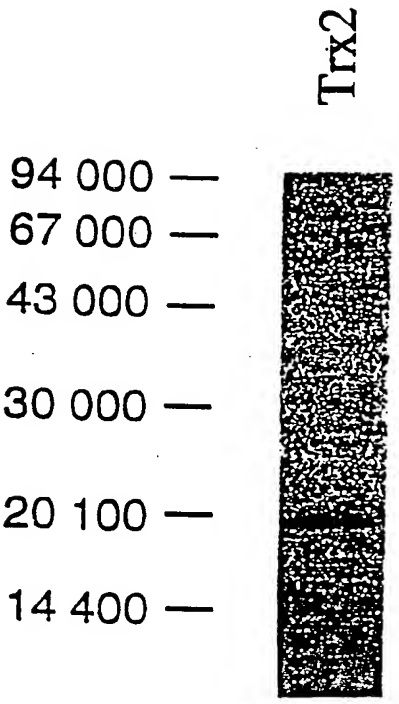
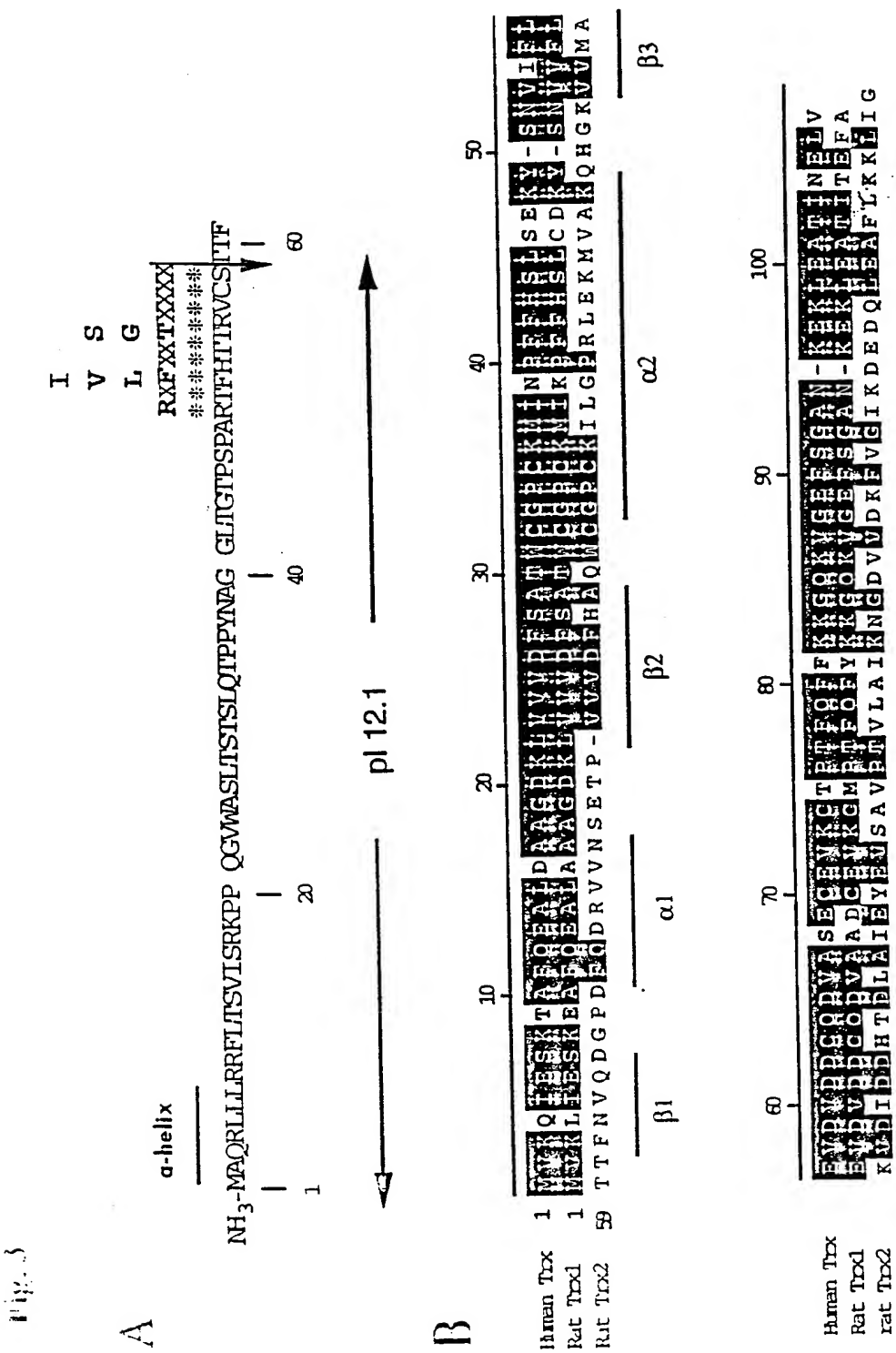
140
160
Regions
Alpha
Beta
Turn
Coil
2.36
0
-1.91
Hydrophilicity Plot

Fig. 2




A
α-helix
NH3-MAQRLLLRRFLITSVLSRKPP QGVWASLTSISLQTPPYNAG GLITGTPSPARTFHTTRVCSTTF
I
V S
L G
RXFXXTXXXX
pI 12.1
B
Human Trx
Rat Trx1
Rat Trx2
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α1
β2
α2
β3
α3
3₁₀
β4
β5
α4

Fig. 4

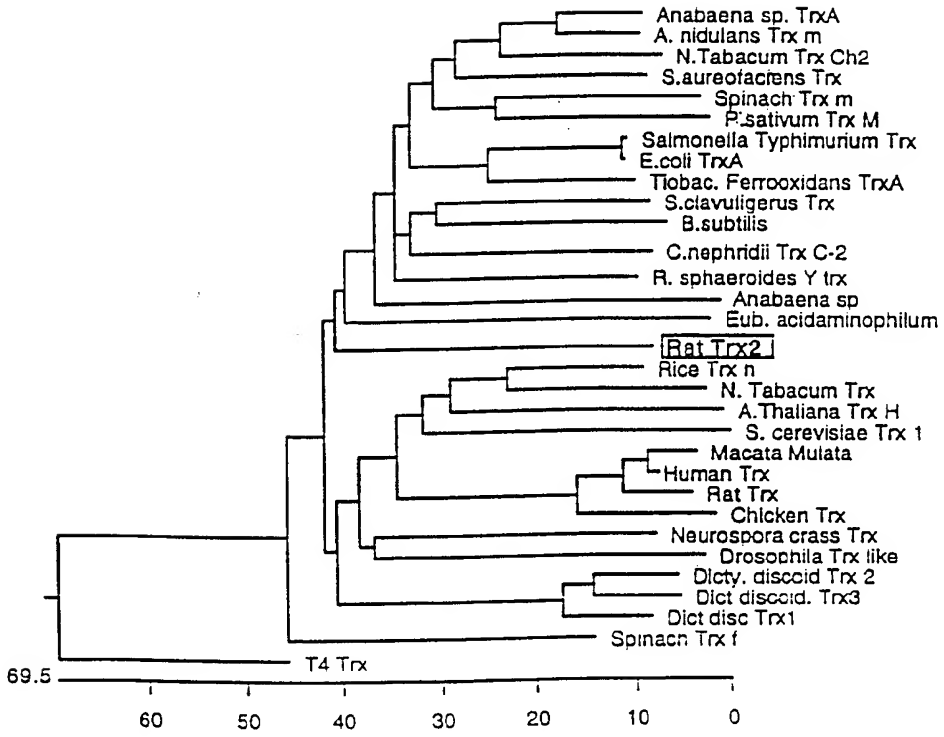

Anabaena sp. TrxA
A. nidulans Trx m
N.Tabacum Trx Ch2
S.aureofaciens Trx
Spinach Trx m
P.sativum Trx M
Salmonella Typhimurium Trx
E.coli TrxA
Tiobac. Ferrooxidans TrxA
S.clavuligerus Trx
B.subtilis
C.nephridii Trx C-2
R. sphaeroides Y trx
Anabaena sp
Eub. acidaminophilum
Rat Trx2
Rice Trx n
N. Tabacum Trx
A.Thaliana Trx H
S. cerevisiae Trx 1
Macata Mulata
Human Trx
Rat Trx
Chicken Trx
Neurospora crass Trx
Drosophila Trx like
Dicty. discoid Trx 2
Dict discoid. Trx3
Dict disc Trx1
Spinach Trx f
T4 Trx
69.5
60
50
40
30
20
10
0

Fig. 5

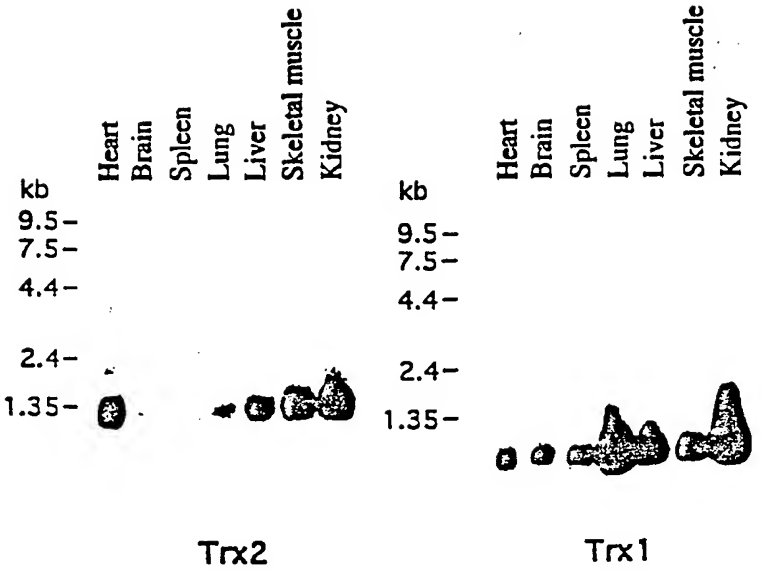

Heart
Brain
Spleen
Lung
Liver
Skeletal muscle
Kidney
kb
9.5
7.5
4.4
2.4
1.35
Trx2
Trx1

Fig. 6

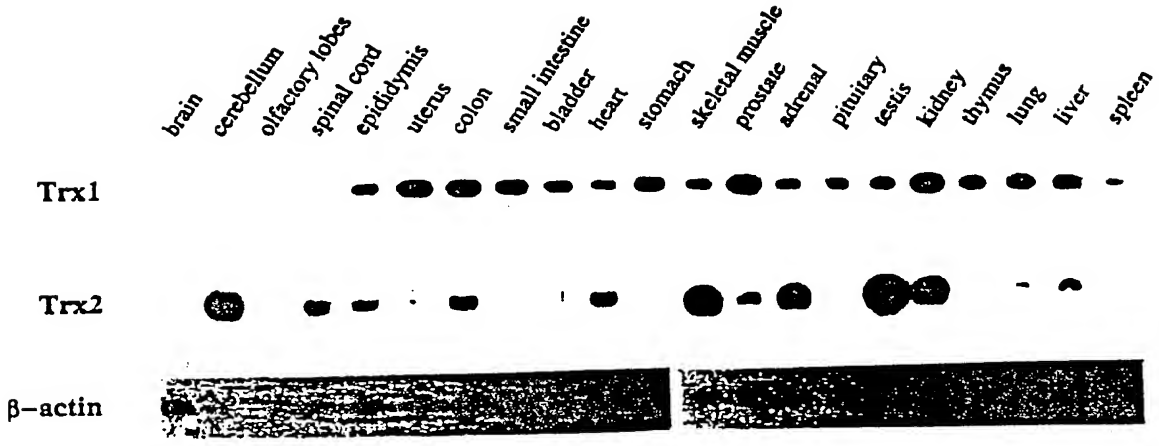

brain
cerebellum
olfactory lobes
spinal cord
epididymis
uterus
colon
small intestine
bladder
heart
stomach
skeletal muscle
prostate
adrenal
pituitary
testis
kidney
thymus
lung
liver
spleen
Trx1
Trx2
β–actin

Fig. 7

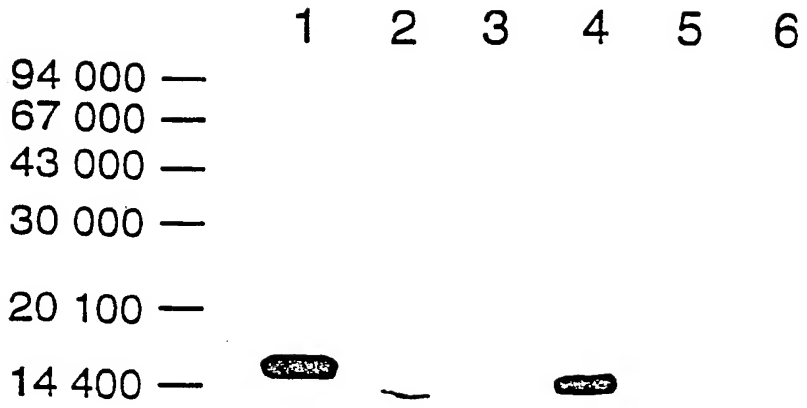


Fig. 8

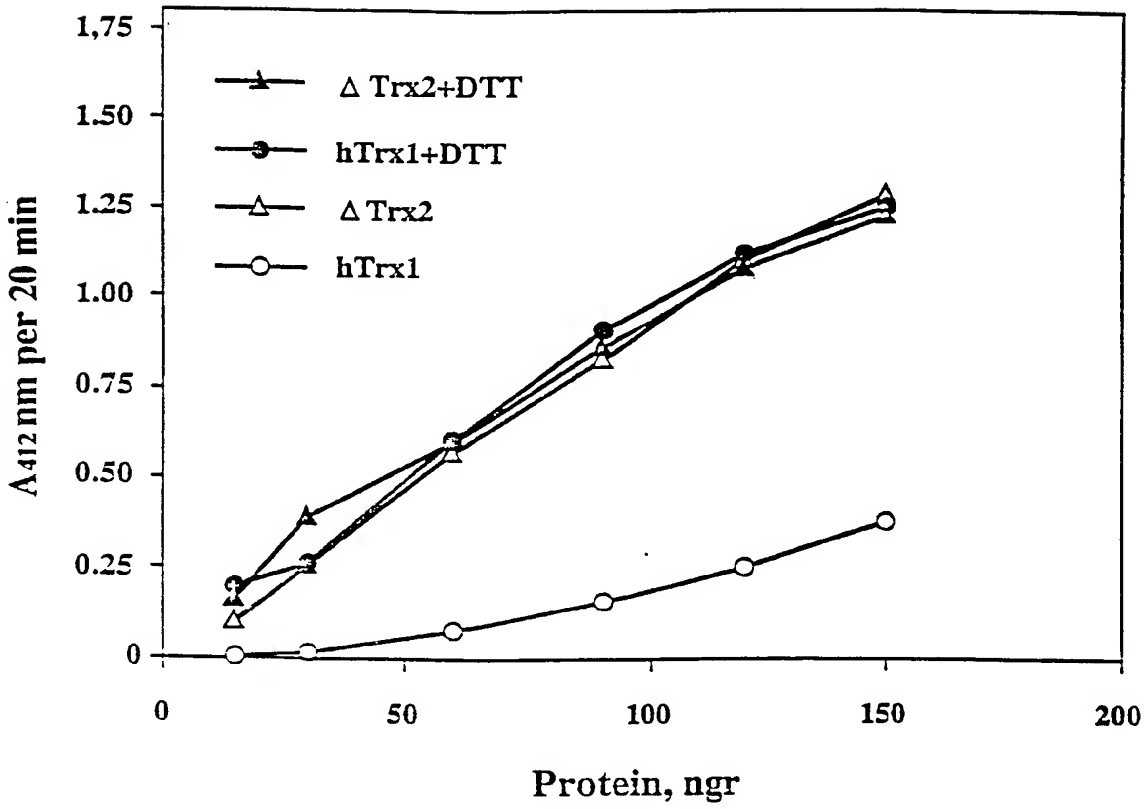
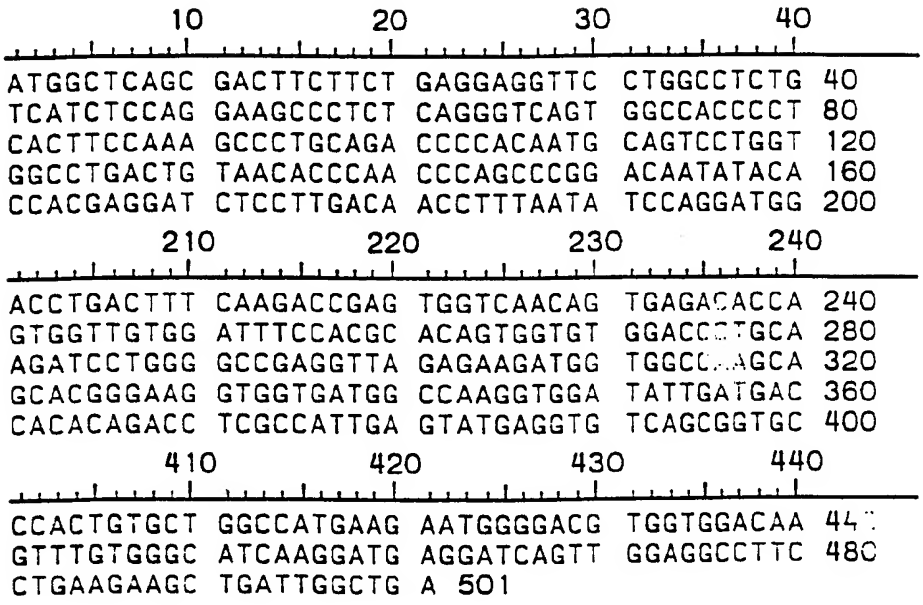

1,75
1.50
1.25
1.00
0.75
0.50
0.25
0
A412 nm per 20 min
Δ Trx2+DTT
hTrx1+DTT
Δ Trx2
hTrx1
0
50
100
150
200
Protein, ngr

Fig. 9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20
30
40
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CACTTCCAAA GCCCTGCAGA CCCCACAATG CAGTCCTGGT 120
GGCCTGACTG TAACACCCAA CCCAGCCCGG ACAATATACA 160
CCACGAGGAT CTCCTTGACA ACCTTTAATA TCCAGGATGG 200
210
220
230
240
ACCTGACTTT CAAGACCGAG TGGTCAACAG TGAGACACCA 240
GTGGTTGTGG ATTTCCACGC ACAGTGGTGT GGACCCTGCA 280
AGATCCTGGG GCCGAGGTTA GAGAAGATGG TGGCCAAGCA 320
GCACGGGAAG GTGGTGATGG CCAAGGTGGA TATTGATGAC 360
CACACAGACC TCGCCATTGA GTATGAGGTG TCAGCGGTGC 400
410
420
430
440
CCACTGTGCT GGCCATGAAG AATGGGGACG TGGTGGACAA 44
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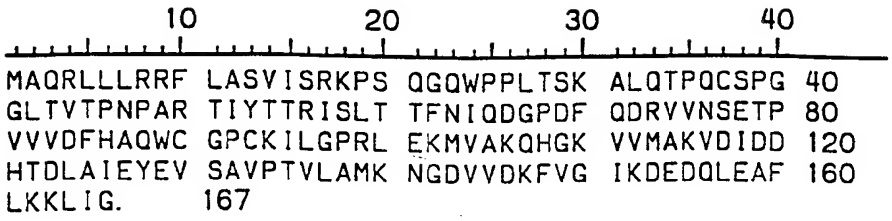
CTGAAGAAGC TGATTGGCTG A 501

Fig. 10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20
30
40
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GLTVTPNPAR TIYTTRISLT TFNIQDGPDF QDRVVNSETP 80
VVVDFHAQWC GPCKILGPRL EKMVAKQHGK VVMAKVDIDD 120
HTDLAIEYEV SAVPTVLAMK NGDVVDKFVG IKDEDQLEAF 160
LKKLIG. 167

Fig. 11

1.

GGGCCCAAAAAAAGAGATCTCTCGAGGATCCGAATTCCGGATCCTGGCCAAGGCAGGAGGAAGTCACCCGTCATAACATG
GGGATTCTGAGGATCTGTGGTGGGCTTTTCACAGTTCCACCCTCTAAGACATATGTAGATATCACACATTGCAGAACCAA
AGGAAGTGGGAAGGTGGTGTAGCCAAATGCTGAAAGCAGTGACCATAAAAAACTAAGTTCAGCCCTTACAGGAACTCAAA
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TGGGCCCTGACCACACCTGGAAGACTTCTCAGGGTTAGAAAGCAGAGAGGCCTGTACACTCATTTTAGTTTACCCCAAGT
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TCCCCTTTNNTNAAGGANTCTTNTTCCATNCCATCCAAAAAATTTTTTTTACTGATTTGCCTACTCCATNCCAAGANAATT
GGTTGGGATGGTTNCCCTCAATACCACCTNNAATTCCAANGCCATGTCTCTCCAACTTTATTCCATTGGGNCCAAGGGNT
GGTTAGGNTAGGTAAAGAAAGCTCCCTTTGGAGGCCAATTGGGGCTTGGAAGGTTNGGGAAAGNCTCCTTANAATTTTTT
TTGGGNAAGGGGGCANGTTGATATCNAAGTTANTTGGAAAAGGAAGGANTNTCC

2.

CAGGGAAAAGAGACTCTCGAGGATCCGAATTCCGTATAAATGACTGAATGAGGCTCTTCAACAGCTGAGTAGCGTGAACT
AGGAAAATTNCANNGGGGNNAATTCTAACATCATTGTTGAACTAGATTGTTTAGATGGGTACTATACCATAGTATGACTA
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GATACAAAAATCAGCTGGGCATGGAGGCAGGTGCCTGTAATGCCAGCCACTCAGGAGGCTGAGGCAGGAGAATCACTTGA
ACCCCGGAGGCGGAAGTTGCAGCGAGCTTCAGATCGANCCACTTNCCCTCCAGGCNGGCAAACAAGAGTGAAAACTCCAT
CTCAAAAAAAANAAGTTATTTCAATGGNATCTCATTTAGCGCAANCTTTCANNTNANTTAAAAATTTATTAAAAATCACT
CNCCCACNCGGGAAAAGAACAAGNTTGGGGTTCTTGATCATCACTCCACTGATGATAATTGGNTTCTTCANCCNCANCTT
CCCGAATCCAGACTTTTTATCNNNGGTTNTCNANACCCCCANNTCNCTNCANTTNNGNTNGNNACAACTNATNTCTTTCN
ATCTTCANCCCNGGCCNCTNCTCCTACTNT

Fig. 12

1.

TCATCTCTCAGTCCCTGCACAGAAATGAGAGATGAACAAAAACGAGACACTTCCACCTGGCCAGAGTCTGGGAGGGCAGG
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CTCACTCCCAGAAGTCATTACACACTTCAAACTGTTCCAAGGAACCCCCTCCCCCATTACTTCTATCCTCACTTCCCACTN
AAAACCAACCNCCAAATTGCCCCCTGCCCAGATCCAACTCCAGAACNATNGCAGTATGGCCATCCTGTCTGGGTGACCTCC
CAAAACATTTCNGGGAT

2.

AGTTCATAATCCTGGTCCAGGCACTGTATGCATCGAATATATTGTCTCATGAGCTTCCTAATGACACGACCTTCCTAGCT
CCTTGTGAATACTATACTATGCAGATCCCTCAGAAGCATGGGACCTGGGACTGGTGGCACCAACTGAGGTGACATTCTCT
GCCAATGTCAGCTTGATTTAGATGTCACTGAATCAAGCTGCTTCCGCCCTGTGTTCCTGTGAATCTGCTCTTGGCTGGCT
TTCTCTGGTGTTGCCTGCTTGCTTTGTTGCTTTGGAAGGCTATCCAAGATATGTAAGCAATTCTTTAGGAAA
TAAATTGCTGTTAACCCAAACAAAACACTGCAGACGAAATGGGTTAAAGGCGAANCAGTGGGGGCGTTGACTAGG
ACAGATGGCTANGGTCTACATGAACTGGGGGAAATTCTTTATGAAATATGTCAACTTTGTGATCAAATGATGTTGATATAA
AATTGTNTAAAGGGAATAATTGTCTGATACTTATCTGTTTGAAAAGTACTCCAGAANCATCTTGATTTAATTATTTAAAG
TTCCTAAAATATCCCCCCCCTNGCTGTTTACNAACTGTTTTATCCCCAGTTGGTGCCTTATGTCCCCATTAAATTGTNTCC
NCCATGATCNTACCTNAAATGAATTT

3.

CTGCTCAGAGCAAGAACTGTTACGAAATGACAGGTATCAAGTGCTGGGTAATATTCATGCATTTTAATTGAACACCCAGG
AAATTTGAGAAATAAAGATGAGAAGAGAAAACTTTAAAAATGCCCCCATAGAGAAGCCCTAGCCTTGATTTTTTTAAGAGAT
TACCTTGCATTGCGGTGGAAACATAAAAATCTCTCGTAGAATGTTTTAATGTGGAAGTTAATATTTACATACTTACAATT
GTGTCTCCCTGAGAAAATCTTTGGTACTGTCAGAAATAGTAAACAGATTCTTTAAGGGCTCANGTAAAGAAGCTGTAGAA
AAGTCCAGAAAAAGACTGGGTACGATTCAGAGGGTNGGTACCTGATTCANAAAGAGAGGGANAGAATTAAGAAGGGAAAAA
AATTAGTGTNAAATGAGCAAGAAATAGTAAGTCTCAAACACAACNGACAAATTCCCACTTGGGCAGAAGAACAGTCATCTTA
GTAATCACAGANAANTGAACCTTNGGGATGTNAATGACCCTTGGCTGGANGACAAGGGTTGAAAATTTGCCCTCCCCATT
CCCTGTTGCAGCAGAATANCGTATGAAATGTTAAGTGAACTGAGGGCCAGGGTTGANACAACCCATTCCATCCTGACCCC
ACTTTGGCCATATACTGATACC

4.

CATGTATGTTCAGTCACATATGCCAAGGCCAAAGAAGATGCCCTGCTTTGTGTCTCTCTACCTTATTCCTTTAGAAAGGG
CTGTTTCTGCACATGGAGGTCTGCTAGCAGCCAGTTCCTTTCTCTCTCTACCCTAGGGTTACAGGTAGGTAGCCATGGCT
ACACCCTACTCTTTATGTGAGTGCTGGAGATTCTGGAAAANCTCCCTATGCTTGTGTGGCTAGCACACTCACCCACTAAG
CAAGCCATCTATCTTGCAAGTCAGCATTTTGATTTTCTTCTATGAAGCCTATCAGGAGGGAAGGTGCAATTAAAAAAAA
TCTATTATATCTTAGGTCATTTTGATCCTACAATCCAAAGCTATGAAATGAACTTGGATTCTTTTCGTTATTCAAACAC

5.

AAACGGCCGCGGTATCCTGACCGTGCAAAGGTAGCATAATCACTTGTTCCTTAATTAGGGACTAGAATGAATGGCTAAAC
GAGGGTTCAACTGTCTCTTACTTTCAATCAGTGAAATTGACCTTCCAGTGAAGAGGCTGGAATCTCCCAATAAGACGAGA
AGACCCTATGGAGCTTTAATTTACTAGTTCAACTTATATAAAAAACAACCTAATGGGCTAAAACAAAATAAATATGAACTA
AAAAATTTCGGTTGGGGTGACCTCGGAGAATAAAAAATCCTCCGAATGATTTTAACCTAGACTCACAAGTCAAAGTAATA
CTAATATCTTATTGACCCAATTATTGATCAACGGACCAAGTTACCCTAGGGATAACAGCGCAATCCTATTTAAGAGTTC

Fig. 13

```
ATG GCT CAG CGA CTT CTT CTG AGG AGG TTC CTG GCC TCT GTC ATC TCC AGG AAG
1
CCC TCT CAG GGT CAG TGG CCA CCC CTC ACT TCC AAA GCC CTG CAG ACC CCA CAA
TGC AGT CCT GGT GGC CTG ACT GTA ACA CCC AAC CCA GCC CGG ACA ATA TAC ACC
ACG AGG ATC TCC TTG ACA ACC TTT AAT ATC CAG GAT GGA CCT GAC TTT CAA GAC
CGA GTG GTC AAC AGT GAG ACA CCA GTG GTT GTG GAT TTC CAC GCA CA: GTGAGTA
                                                          88
TT...INTRON 1 (4kb)...GCCTTTTGAG :G TGG TGT GGA CCC TGC AAG ATC CTG GGG
                                  89
CCG AGG TTA GAG AAG ATG GTG GCC AAG CAG CAC GGG AAG GTG GTG ATG GCC AAG
GTG GAT ATT GAT GAC CAC ACA GAC CTC GCC ATT GAG TAT GAG :GTATGGATTGA...
                                                    109
INTRON 2 (9kb)...TCCACCCAG: GTG TCA GCG GTG CCC ACT GTG CTG GCC ATG AAG
                           110
AAT GGG GAC GTG GTG GAC AAG TTT GTG GGC ATC AAG GAT GAG GAT CAG TTG GAG
GCC TTC CTG AAG AAG CTG ATT GGC TGA CAAGCAGGGATGAGTCCTGGTTCCCTTGCCCGCGT
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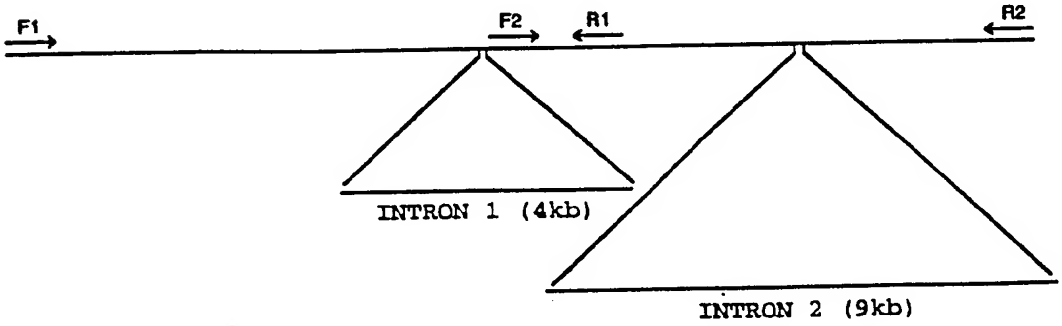


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Primer F1: 5'-GATGGCTCAGCGACTTCTTC
Primer F2: 5'-GGTGGTGTGGACCCTGCAAGATC
Primer R1: 5'-CCACCTTGGCCATCACCACCTTC
Primer R2: 5'-GGCAAGGGAACCAGGACTCATCC
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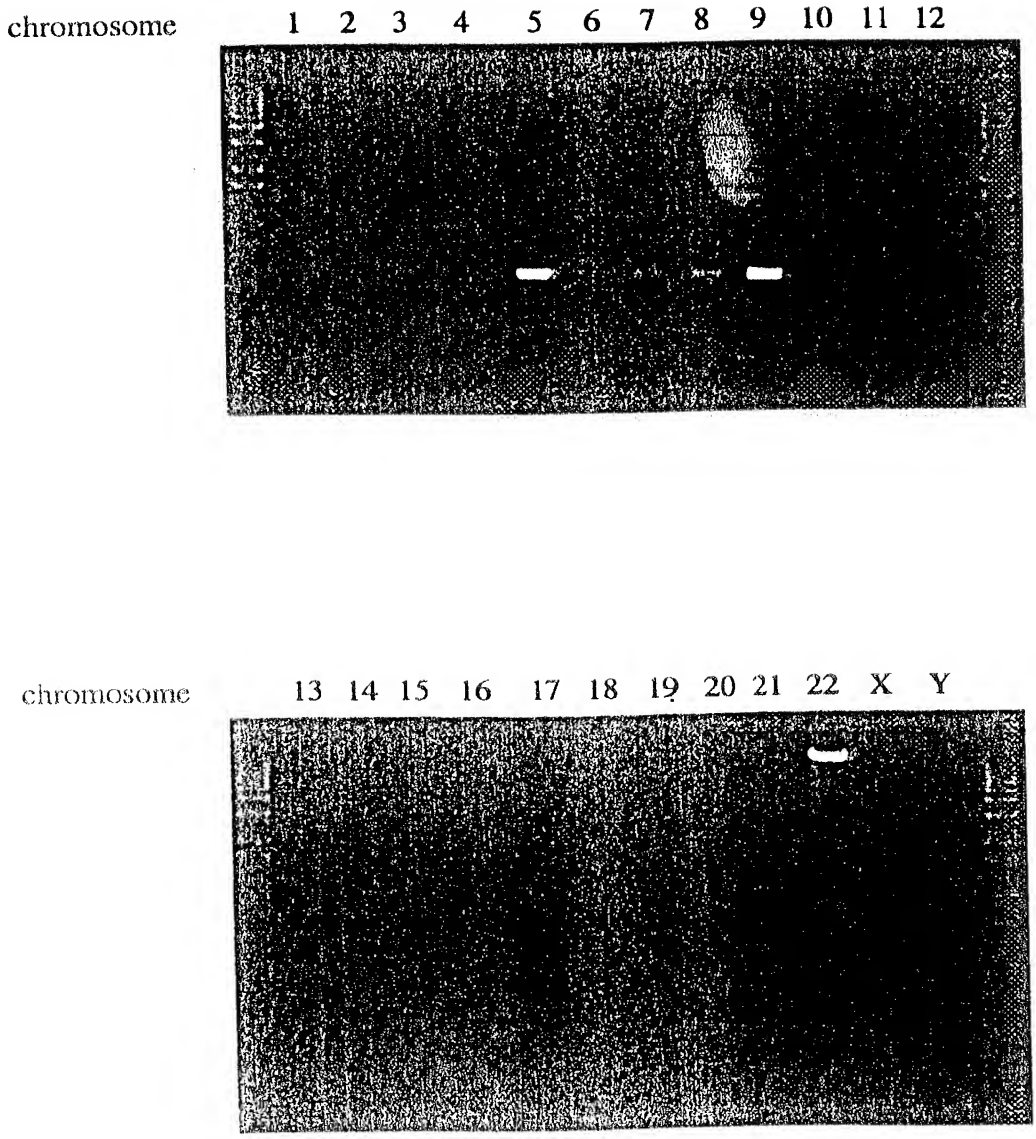
FIG. 14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1 2 3 4 5 6 7 8 9 10 11 12
chromosome
13 14 15 16 17 18 19 20 21 22 X Y

FIG. 15

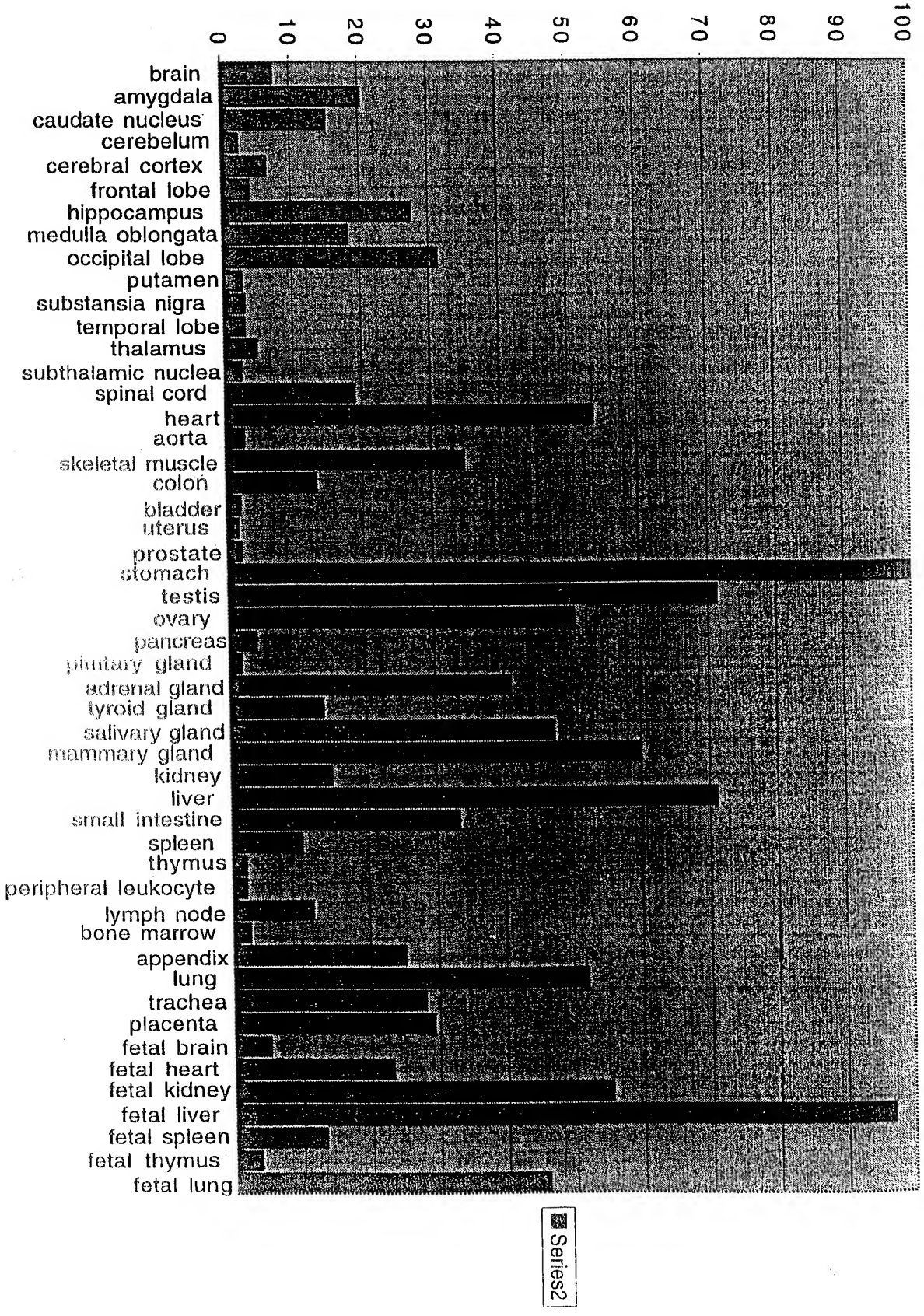
0
10
20
30
40
50
60
70
80
90
100
brain
amygdala
caudate nucleus
cerebelum
cerebral cortex
frontal lobe
hippocampus
medulla oblongata
occipital lobe
putamen
substansia nigra
temporal lobe
thalamus
subthalamic nuclea
spinal cord
heart
aorta
skeletal muscle
colon
bladder
uterus
prostate
stomach
testis
ovary
pancreas
pituitary gland
adrenal gland
tyroid gland
salivary gland
mammary gland
kidney
liver
small intestine
spleen
thymus
peripheral leukocyte
lymph node
bone marrow
appendix
lung
trachea
placenta
fetal brain
fetal heart
fetal kidney
fetal liver
fetal spleen
fetal thymus
fetal lung
Series2

Fig. 16